ALTERATION OF BIOMASS COMPOSITION IN RESPONSE TO CHANGING SUBSTRATE PARTICLE SIZE AND THE CONSEQUENCES FOR ENZYMATIC HYDROLYSIS OF CORN BRAN

Jane Agger and Anne S. Meyer*

Corn bran is a by-product from corn starch processing. This work examined the effects of changing substrate particle size on enzymatic hydrolysis of both raw and pretreated destarched corn bran. The biomass composition of the corn bran varied between particle size fractions: The largest particles ([1000;710]µm) were richer in cellulose and in (arabino) xylan with a relatively low degree of arabinofuranosyl substitutions, whereas the smaller particles ([250;150]µm) contained less cellulose, but arabinoxylan with higher arabinofuranosyl substitution (higher A:X ratio). Enzymatic hydrolysis yields improved with decreasing substrate particle size, particularly for the raw corn bran. The increased enzymatic yields obtained with decreasing substrate particle sizes were related to the increased substrate surface area but also to the biomass composition. Theoretical estimations of enzymatic reaction efficiency supported that biomass composition affected the enzymatic reaction yields and provided new insight into the impact of substrate particle size on enzymatic biomass hydrolysis.

Keywords: Corn bran; Arabinoxylan; Particle size; Enzymatic deconstruction; Biomass; Lignocellulose composition

*Contact information: Center for BioProcess Engineering, Department of Chemical and Biochemical Engineering, Technical University of Denmark (DTU), DK-2800 Kgs. Lyngby, Denmark; *Corresponding author: am@kt.dtu.dk*

INTRODUCTION

Corn bran is a co-processing product from the corn starch wet-milling process and constitutes an abundant, readily available agro-industrial residue. Corn bran represents the tough and resistant outer layer of corn kernels and is rich in C-5 and C-6 carbohydrates that are potentially interesting substrates for upgrading to be used in food and fuel products (Agger *et al*. 2010; Appeldoorn *et al.* 2010). Milled corn bran mainly consists of primary cell walls from the pericarp of corn kernels and possibly also the pedicel tip of the kernels, testa, and some residual starch. Arabinoxylan, cellulose, and starch make up the main constituents of corn bran (Agger *et al*. 2010; Appeldoorn *et al.* 2010; Saulnier *et al*. 1999). The arabinoxylan polysaccharides of corn bran are very complex with respect to structure and composition, and corn bran is exceptionally insubmissive to enzymatic degradation (Agger *et al*. 2010; Faulds *et al.* 1995; Saha and Bothast 1999; Saulnier *et al.* 2001).

Arabinoxylan principally consists of a β-D-(1→4) linked xylan backbone with various substitutions. A particular feature of arabinoxylan is the extent and positioning of the α-L-arabinofuranosyl substitutions on the xylan backbone. Corn bran arabinose-to-xylose ratio, abbreviated A:X ratio, has been reported to be in the range of 0.6 to 0.7 (Agger *et al*. 2010; Chanliaud *et al.* 1995). This ratio is close to that of wheat endosperm arabinoxylan, for which the A:X ratio is typically around 0.6 irrespective of whether the arabinoxylan is soluble or insoluble (Sørensen *et al.* 2007). Corn bran arabinoxylan may moreover be decorated with L- and D-galacto-pyranosyls, acetyl, coumaryl, feruloyl, and diferuloyl residues, the latter even cross-linking arabinoxylan polymers (Saulnier *et al*. 1995a; Bunzel *et al.* 2001; Chesson *et al.* 1983). Glucuronic acid is also known as a common component in arabinoxylan and recently has been claimed to be associated with the xylan oligomers in corn bran (Appeldoorn *et al.* 2010).

Efficient enzymatic deconstruction is dependent upon the amenability of the substrate to enzymatic attack. In order to enhance enzymatic conversion, most cellulosic biomasses are therefore hydrothermally pretreated in a separate step prior to the enzymatic treatment (Agger *et al*. 2011; Pedersen and Meyer 2010). Several studies have examined the effects of diminishing substrate particle size prior to such pretreatment or prior to the enzymatic hydrolysis of lignocellulosic biomass materials (Chandra *et al.* 2007; Chundawat *et al.* 2007; Mooney *et al.* 1998; Pedersen and Meyer 2009; Zeng *et al.* 2007). Increased enzymatic degradation in response to biomass particle size diminution has usually been interpreted as being directly related to increases in the substrate surface area, which in turn creates higher enzymatic accessibility to the substrate. Differences in biomass composition among varying particle sizes, however, have been observed for different cellulose and xylan substrates, and it has also been shown that differences in particle size may inherently sort the material to originate from different plant tissues (Chundawat *et al.* 2007). It is thus known that enzyme-catalyzed cellulose and xylan degradation are not affected equally by particle size reduction (Alvo and Belkacemi 1997; Chundawat *et al.* 2007; Pedersen and Meyer 2009). The particle size reduction resulting from the enzymatic degradation itself has been suggested to contribute to enhance the enzymatic hydrolysis during intense mixing of cellulose fiber suspensions (Samaniuk *et al.* 2011), but Sinitsyn *et al.* (1991) found that enzymatic hydrolysis on crystalline cellulose was not significantly affected by increasing the specific substrate surface area. These types of results have not received appropriate attention. Notably, differences in biomass composition produced as a result of substrate particle size reduction have hardly been addressed. The overall purpose of this work was to enhance the enzymatic degradation of corn bran to produce high yields of monosaccharides as a prerequisite for utilization of corn bran in fermentation processes. In this regard, the objective of the work was to examine the monosaccharide composition of corn bran substrates having different particle sizes and to evaluate whether particle size and/or any possible compositional differences affect the enzymatic hydrolysis evaluated as enzymatic monosaccharide yields. It was hypothesized that the structural composition and content of non-starch polysaccharides in corn bran would not necessarily be uniform in all particle size fractions and that any increasing enzymatic yields correlating with decreasing particle size would not necessarily be a result of increased substrate surface area only, but also be influenced by biomass composition.

EXPERIMENTAL

Substrate

Corn bran was obtained from Archer Daniels Midlands Co., Decatur, IL, USA as the by-product from corn starch wet-milling. The material was destarched with α-amylase and amyloglucosidase in a two-step process as described previously (Agger *et al.* 2010). The destarched corn bran is referred to as DCB. The destarched corn bran was pretreated using heat at the Danish National Laboratory for Sustainable Energy, Roskilde, Denmark as described previously (Agger *et al.* 2010). The pretreatment encompassed heating of a 6% (w/v) aqueous slurry, having a pH *ca.* 5.5 to 6, in a loop autoclave at 190 ºC for 10 min (Bjerre *et al.* 1996). No other chemicals were added. After pretreatment, the pH was 4.2 in the total slurry. The pretreated DCB was separated into soluble and insoluble fractions by filtration, and the insoluble residue was washed in Milli-Q water and freeze dried.

Substrate Particle Size Reduction and Sieving

Particle size reduction was achieved by processing the material through a Retsch SM 2000 cutting mill with a 0.5 mm screen. After each particle size reduction step, the material was sieved using analytical sieves with apertures of 1000, 710, 355, 250, and 150 µm (Endecotts, London, UK). Further particle size reduction of the DCB fraction [1000;710] µm was achieved by the use of a benchtop cutting mill Retsch ZM 100 with a 0.5 mm screen, and subsequent sieving of the milled material using the analytical sieves (Endecotts, London, UK) as described above. The mass distribution of each particle size fraction was obtained by the use of a laboratory balance. The sieving resulted in 4 particle size fractions, designated as: [1000;710], [710;355], [355;250], and [250;150] µm.

Determination of Substrate Composition

In order to determine the overall monosaccharide composition of each set of particles, each particle size fraction was subjected to two different kinds of acidic hydrolysis: either 0.4 M HCl for 2 hours at 100 ºC to facilitate the quantification of xylose, arabinose, and galactose (Sørensen *et al.* 2003) or the two-step H_2SO_4 hydrolysis process to quantify cellulosic glucose and Klason lignin according to the standard procedure of the U.S. National Renewable Energy Laboratory (NREL) (Sluiter *et al.* 2008). The Klason lignin was corrected for protein content. Protein content was determined according to the methodology of Barkholt and Jensen (1986) which includes a complete hydrolysis of proteins to amino acids via a 6 M HCl hydrolysis treatment for 24 hours followed by quantification by ion exchange chromatography. The reason for using a protein quantification method based on quantification of individual amino acids was due to the concern that the protein composition in the substrate material might change after high temperature pretreatment.

Enzymatic Hydrolysis Treatment

All size fractions of both substrates were hydrolysed enzymatically as previously described (Agger *et al.* 2010). In brief, the enzyme blend consisted of a designed mixture of monocomponent endo-β-xylanase, β-xylosidase, two α-L-arabinofuranosidases,

feruloyl esterase, acetyl xylan esterase, and a commercial cellulase preparation Cellic™ CTec (Generation 2009) (Agger *et al.* 2010, 2011). Cellic™ CTec is based on the *Trichoderma reesei* cellulase complex (exo-glucanase, endo-glucanase, and β-glucosidase activities) but also contains additional β-glucosidase and glycoside hydrolase family 61 hydrolysis boosting proteins (Harris *et al.* 2010). An overview of the enzymes and dosages is shown in Table 1. All hydrolysis experiments were performed in triplicate at 2% w/v dry matter (DM), incubated for 24 hours at 50 ºC and a pH of 5 in a 0.1 M succinate buffer, and each reaction was stopped by heating to 100 ºC for 10 min.

Table 1. Enzymes Used for Bench Marking of Enzymatic Hydrolysis of the Different Particle Size Fractions of all Three Substrates **

Enzyme	Microorganism	Family	EC no.	Ref.*
Endo-1,4-β-xylanase	*Humicola insolens*	GH10	3.2.1.8	I
β-xylosidase	*Trichoderma reesei*	GH3	3.2.1.37	I
α-L-arabinofuranosidase	*Meripilus giganteus*	GH51	3.2.1.55	I
α-L-arabinofuranosidase	*Humicola insolens*	GH43	3.2.1.55	I
Acetyl xylan esterase(AXE)	*Flavolaschia sp.*	CE1	3.1.1.72	II
Feruloyl esterase(FAE)	*Aspergillus niger*	Type A, CE1	3.1.1.73	III, IV
Cellic™ CTec	*Trichoderma reesei*	-		II, V

* I: (Sørensen *et al.* 2007); II: (Agger *et al.* 2010); III: (Faulds and Williamson 1994); IV: (Faulds and Williamson 1995); V: (Harris *et al.* 2010).
** All enzymes, including the Cellic™ CTec preparation were provided by Novozymes A/S. Cellic™ CTec is a commercially available mixed cellulase based preparation. Glycosyl hydrolases were dosed at 0.25 mg/g DM each, esterases at 0.5 mg/g DM each, Cellic™ CTec at 4 mg/g DM.

Monosaccharide Analysis for Enzymatic Yield Estimation

Monosaccharides arabinose, galactose, glucose, and xylose were analysed and quantified by HPAEC-PAD, on a BioLC Dionex equipped with a CarboPac™ PA1 (analytical 4 x 250 mm) column from Dionex (Sunnyvale, CA). The elution profile consisted of an isocratic pre-run with 25 mM NaOH for 5 minutes, followed by isocratic elution with 10 mM NaOH for 12 min, then by 7 min of regeneration of the column with 500 mM NaOH and 5 min re-equilibration to 25 mM with a flow of 1 mL/min.

RESULTS AND DISCUSSION

Biomass Composition

The general trend for the relative biomass composition in DCB and in the pretreated DCB was that the polysaccharide content and composition varied with varying particle size (Table 2). The xylose and glucose contents decreased with decreasing particle size, whereas the arabinose content seemed to be constant for varying particle size fractions. The galactose content was low and followed the trend of xylose and glucose (data not shown). The xylose and arabinose content trend caused the A:X ratio to increase with decreasing particle size, indicating that the extent of arabinofuranosyl

substitution of the xylan backbone in the smaller particles was greater than that in the larger particles. Since the material had been destarched prior to the experiments, it was assumed that all glucose originated from cellulose and the data thereby signified that the cellulose content was highest in the large particles. The cellulose content in corn bran was in the same range as that reported by others (Saulnier *et al.* 1995b).

Table 2. Biomass Composition of Destarched Corn Bran (DCB) and Pretreated DCB after Sieving into Different Particle Size Fractions; Biomass Composition Data are given as Levels in Each Individual Particle Size Fraction.*

Substrate	Fraction (µm)	Mass distrib. (%)	Xylose (g/kg DM)	Arabinose (g/kg DM)	Glucose (g/kg DM)	A:X	Protein (g/kg DM)	Lignin (g/kg DM)	Sum** (g/kg DM)
DCB	[1000;710]	17	476 ±0.4^{a}	280 ±0.6^{a}	240 ±11^{a}	0.59	48.8 ±0.0	96.2 ±5.2	1141
	[710;355]	52	432 ±9.3^{b}	276 ±7.5^{a}	219 ±19^{ab}	0.64	60.8 ±2.2	116 ±9.8	1104
	[355;250]	20	296 ±7.0^{c}	277 ±3.0^{a}	182 ±19^{ab}	0.94	117 ±1.7	103 ±6.6	975
	[250;150]	9.7	262 ±11^{d}	265 ±9.2^{a}	172 ±9.4^{b}	1.01	163 ±0.0	67.4 ±4.1	929
	Non sieved	-	374 ±0.6	267 ±0.4	233 ±13	0.71	89.1 ±0.3	30.9 ±1.8	994
Pretreated DCB	[1000;710]	12	141±3.2^{b}	59 ±1.6	358 ±2.8^{a}	0.42	118 ±2.8	82.6 ±0.5	759
	[710;355]	53	156±4.4^{a}	66 ±1.0	281 ±22^{b}	0.42	83.6 ±10.0	111 ±9.1	698
	[355;250]	19	124±3.9^{c}	61 ±0.3	181 ±3.5^{d}	0.50	137 ±0.1	126 ±12	629
	[250;150]	13	108±2.7^{d}	56 ±1.0	239 ±1.0^{c}	0.52	139 ±12.7	145 ±5.1	687
	Non sieved	-	139±1.8	65 ±0.9	345 ±1.3	0.47	115 ±10.0	94.4 ±4.5	758
	* Superscripts a, b, c, and d indicate significantly different groups based on ANOVA with pooled standard deviations in a 95% confidence interval. Lignin content was determined as Klason lignin corrected for protein. **Sum: ((Hydrated) Arabinose+Xylose+Glucose)+Protein+Lignin								

The compositional differences between particle size fractions of pretreated DCB were less pronounced than those observed for native DCB (Table 2). This was not unexpected since previous investigations (Agger *et al.* 2010, 2011) have shown that approximately 50% of the original DCB biomass was solubilized during hydrothermal pretreatment and that the solubilized material was mainly composed of highly substituted arabinoxylo-oligosaccharides. The insoluble residue after pretreatment was less substituted than the native and richer in cellulose.

The varying biomass composition between particle sizes must inevitably be a result of a relatively heterogeneous starting material. The milling and sieving have apparently caused a sorting of the material into compositionally different substrates originating from the same material.

Table 3. Biomass Composition of Extra Particle Size Reduced Destarched Corn Bran (DCB) after Sieving into Different Particle Size Fractions. Biomass Composition Data are given as Levels in Each Individual Particle Size Fraction.*

Substrate	Fraction (µm)	Mass distrib. (%)	Xylose (g/kg DM)	Arabinose (g/kg DM)	Glucose (g/kg DM)	A:X	Protein (g/kg DM)	Lignin (g/kg DM)	Sum** (g/kg DM)
Extra size reduced DCB [1000;710]	[1000;710]	-	476 ±0.4^{a}	280 ±0.6^{a}	240 ±11^{a}	0.59	48.8 ±0.0	96.2 ±5.2	1141
	[710;355]	47	427 ±2.1^{b}	287 ±2.2^{a}	267 ±26^{a}	0.67	38.7 ±2.1	15.2 ±4.0	1035
	[355;250]	34	377 ±3.7^{c}	287 ±2.3^{a}	257 ±16^{a}	0.76	61.8 ±0.0	10.2 ±0.5	993
	[250;150]	16	371 ±8.1^{c}	281 ±7.2^{a}	274 ±3.8^{a}	0.76	62.2 ±1.4	21.8 ±0.6	1010
	* Superscripts a, b, c, and d indicate significantly different groups based on ANOVA with pooled standard deviations in a 95% confidence interval. Lignin content is determined as Klason lignin corrected for protein. **Sum: ((Hydrated) Arabinose+Xylose+Glucose)+Protein+Lignin								

The relative content of monosaccharides in the different particle sizes compared to the original non-sieved material (calculated from the data in Table 2) showed variation among particle sizes, and particularly the relative xylose content differed between particle sizes. Hence, the compositions of the non-sieved DCB and the pretreated DCB were apparently not uniform, but seemed to consist of regions with alternating contents and compositions of the structural polysaccharides leading to different physical/mechanical properties for milling. This milling and sorting into different particle sizes resulted in each particle size being relatively more similar than the non-sieved material.

The DCB fraction [1000;710] µm was additionally milled down to smaller particles, sieved again, and each size fraction was then characterized with respect to monosaccharide composition in order to evaluate if the composition would change again. The results show that the glucose content was constant across the different particle sizes and similar to the content in the original [1000;710] µm particles (Table 3). This result differed from that obtained after the first round of milling (Table 2); however, as in the first round of milling, the arabinose content was also constant in the different particle size fractions after re-milling and in accordance with the level in the original [1000;710] µm fraction (Table 3). The xylose content decreased somewhat with decreasing particle size (Table 3), but the differences in xylose content compared to the original [1000;710] µm fraction were markedly smaller (476 to 262 g/kg DM versus 476 to 371 g/kg DM).

These data must imply that the original [1000;710] µm fraction in itself was heterogeneous but relatively less so than the native non-sieved DCB. Again, the additional milling and sieving apparently resulted in an organization of the material into three relatively more similar, (but still) heterogeneous fractions; hence, the data suggest that repetitive milling and sieving can generate more and more similarly composed fractions of biomass, whereas one round of milling and particle size sieving will in effect sort the material into differently composed fractions (of different particle sizes). This conclusion is in complete accordance with previous findings in which biomaterials were

found to sort according to origin and composition after milling and sieving (Alvo and Belkacemi 1997; Chundawat *et al.* 2007).

Possible Bias of the Analytical Methodology

Corn bran is a biological material and it is therefore relevant to consider whether the data in Tables 2 and 3 represent a generic tendency. For these studies, one large batch of destarched corn bran (from approx. 15 kg of raw material) was used, with the raw material originating from an industrial process. Thus, the corn bran used was a sample from a mixture of corn batches over a certain period of time, which would in turn level out data variations caused by batch differences, consequently making these data more reliable.

The analytical methods used to generate the monosaccharide composition have been carefully chosen to suit this kind of biomass. In that sense, xylose and arabinose levels were determined after HCl hydrolysis rather than after H_2SO_4 hydrolysis. This was done since H_2SO_4 treatment has been found to grossly underestimate the two components. The principle of H_2SO_4 hydrolysis is that it swells and disrupts the microfibrillar cellulose structure during an initial acid-concentrated step followed by depolymerisation in a dilute acid step, where only the latter includes internal standards (Sluiter *et al.* 2008). The initial acid step would degrade the acid labile pentose from hemicellulose without the chance of estimating a recovery, thereby causing underestimation. Furthermore, HCl has been found to catalyse less formation of degradation products from xylose and arabinose than H_2SO_4 (Lavarack *et al*. 2002). In the method employed here, internal standards were moreover included during the entire HCl hydrolysis. Lignin was determined as Klason lignin after H_2SO_4 hydrolysis and corrected for protein content in each fraction as proteins would create an artificial overestimation of lignin by adding to the insoluble residue after hydrolysis.

The sum of components in Tables 2 and 3 does not account for the loss of water from monosaccharides when in a polymeric form (since the polysaccharides chain length and extents of substitutions are unknown). This methodology may have tended to overestimate the components in the larger particles and possibly underestimate the content in the smaller particles. Particularly in the case of the pretreated DCB, the sums do not account for the entire mass (Table 2). This result may be a consequence of losses encountered due to degradation products formed during the hydrothermal pretreatment; there products could solubilize during the acid hydrolysis and thereby not be a part of the quantified products or residuals.

In addition, as observed previously for wheat straw (Pedersen and Meyer 2009), the ash content of the smaller particles was 2 to 4 times larger than that in the larger particles in both pretreated and unpretreated DCB (data not shown), which may have contributed to the decrease in mass balance closure; however, these shortcomings do not alter the interpretation that milling and sieving apparently result in an organization of the material into differently composed fractions – in addition to sorting it into different particle sizes, and that, in this case, the repetitive milling and sieving of the corn bran material, gradually sorted the material into three more and more similar, (but still) heterogeneous fractions.

Recovery Estimation

The components in the different size fractions can be assumed additive, and based on the relative mass distribution and the data in Tables 2 and 3, the analytical accuracy was assessed by comparing a calculated weighed average composition to the original measured composition in the non sieved material (Table 4). From here, the xylose and arabinose contents in the sieved fractions were found to correspond well with the total content in the non-sieved material, whereas the glucose and protein contents in both the DCB and the pretreated DCB seemed slightly underestimated (Table 4).

The lignin content in the DCB, however, was either vastly overestimated in the sieved fractions or underestimated in the original material. The discrepancy (Table 4) is most likely related to the nature of Klason lignin determination, as it is defined as the insoluble residue after hydrolysis and thereby not quantified as a specific compound. Also, the lignin only made up about 40 g/kg DM of the original material; hence even small deviations in the estimations created large discrepancies in the balance.

Table 4. Mass Recovery Estimations: Comparison of the Originally Analyzed Composition and Calculated Weighed Average Composition[1] of Non-sieved DCB and Pretreated DCB Based on the Mass Distribution and Data in Table 2.*

Substrate		Xylose	Arabinose	Glucose	Protein	Lignin
DCB	Non sieved original (g/kg DM)	374	267	233	89.1	30.9
	Weighed average (g/kg DM)	390	272	208	79.1	104
	Balance	104%	102%	89.1%	88.7%	336%
Pretreated DCB	Non sieved original (g/kg DM)	139	65	345	115	94.4
	Weighed average (g/kg DM)	137	61	258	103	112
	Balance	98.6%	93.4%	74.7%	89.6%	118%

* A Balance (%) of 100 represents complete compliance between measured and calculated weighed average. Balance (%) above 100 indicates overestimation by weighed average and Balance (%) below 100 indicates underestimation. [1]The weighed average calculated as the quantity sum in each fraction ($C_{component,fraction}$) times the mass distribution (X%) from Table 2: $\sum X\% \cdot C_{component,fraction}$

Generally, the balances (Table 4) inform that the hydrolysis methods employed were reproducible and that the variations between biomass compositions thereby represented true variations, even though the methodology has disadvantages. Biomass compositional changes in response to changing particle size are rarely properly acknowledged to explain variation in enzymatic saccharification efficiencies, and only a few papers have recognized such biomass composition differences in relation to

enzymatic hydrolysis efficiency (Alvo and Belkacemi 1997; Pedersen and Meyer 2009; Zeng *et al.* 2007).

Enzymatic Hydrolysis

Enzymatic hydrolysis of each particle size fraction of DCB and pretreated DCB showed that the enzyme catalyzed release of monosaccharides increased with decreasing particle size (Fig. 1).

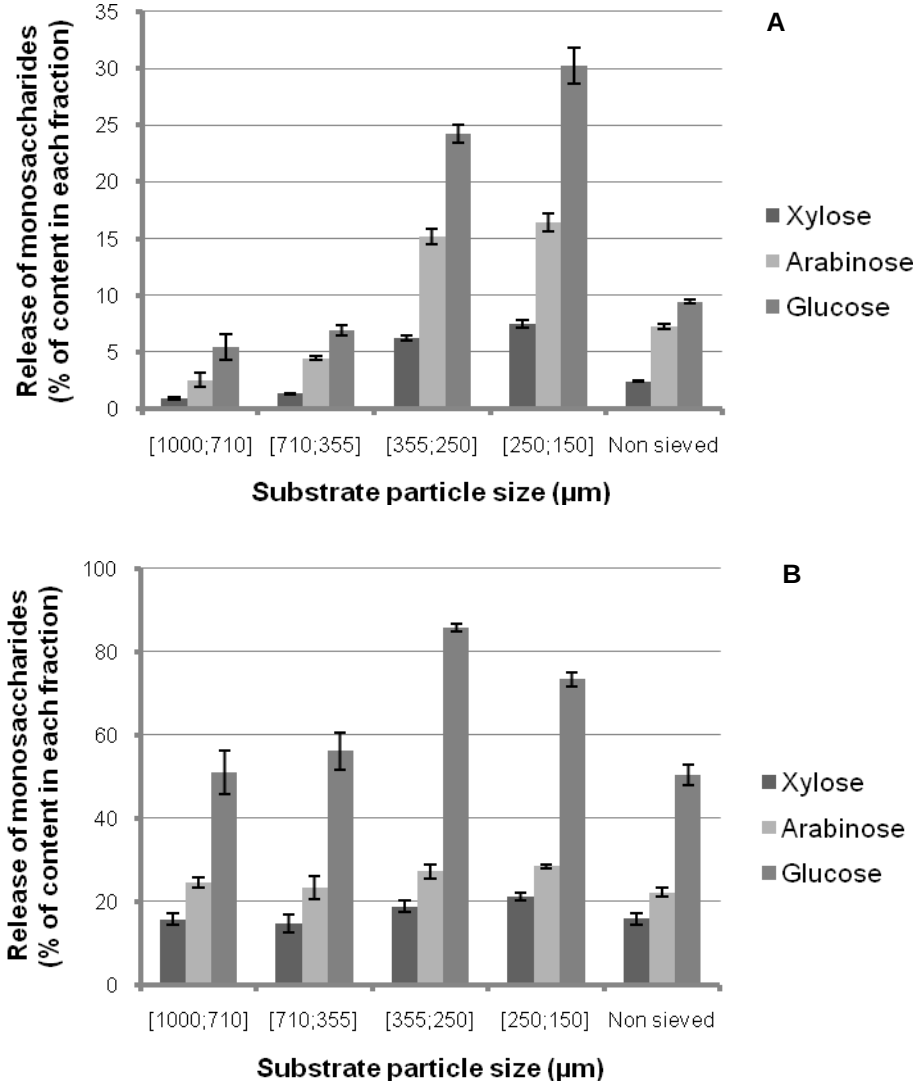

Fig. 1. Enzymatic release of monosaccharides from different particle size fractions after treatment with defined enzyme mixtures (Table 1). A: Release from DCB. B: Release from pretreated DCB. Note the differences in y-axis scales.

The largest relative effect of reducing the particle size was observed for DCB, whereas the overall quantitative release of monosaccharides was largest for pretreated DCB (Fig. 1). These findings are in complete accordance with previously published data

that showed that pretreatment promoted enzymatic hydrolysis, but at the same time leveled out differences observed between different particle sizes (Alvo and Belkacemi 1997; Pedersen and Meyer 2009). One obvious explanation for the increase in the extent of enzymatic hydrolysis was the increased substrate surface area resulting when the substrate particle size was reduced, leading to higher substrate accessibility. The impact of reducing the substrate particle size on hydrolysis yields for DCB, however, was somewhat larger than anticipated, inspiring the thought that other parameters than the substrate surface area were influencing the extent of enzymatic hydrolysis. The differences in biomass composition between particle sizes were also larger for the DCB than for the pretreated DCB, thus suggesting a correlation between the extent of hydrolysis and biomass composition.

Evaluation of increasing surface area could also be done by comparing the arabinose release to glucose release from pretreated DCB. For arabinose, the yields were more similar between the different particle sizes as compared to especially glucose release, which indicated that the release of arabinose from pretreated DCB was less dependent on changes in surface area.

It would be expected that in the case that changes in surface area were the major limiting factor for enzymatic hydrolysis, the observed effects of all three components would be equally affected, but this was not the case. At the same time especially arabinose content was relatively constant between the particle size fractions; therefore, it seems plausible that the increasing enzymatic yields with decreasing particle size were caused not only by an increase in surface area but also as a response to the particular biomass composition in each fraction. This idea is novel as compared to previous conclusions drawn from promotion of enzyme catalysis by particle size reduction, where traditionally, only available surface area and pore volumes have been addressed (Mooney *et al.* 1998; Mansfield *et al.* 1999); however, it ought not to be a surprise that biomass composition influences enzymatic hydrolysis.

The implications of this idea is also that the increasing complexity of arabino-xylan by the increasing A:X ratio is not necessarily retarding the enzymatic hydrolysis. This contrasts the conception that increasing the complexity of arabinoxylan poses a hindrance to enzymatic hydrolysis; however, in the present work, enzymes specifically targeted towards catalyzing the removal of arabinosyl residues from both singly and doubly substituted xyloses were used (Table 1) (Sørensen *et al.* 2006; 2007). The use of this particular enzyme blend might have therefore disregarded the barriers of extensive arabinosyl substitutions.

Cellulose composition should be somewhat comparable between particle sizes, but the content and possible interactions with arabinoxylan might still be different (Fig. 1). Comparing the glucose release from DCB to the glucose release from pretreated DCB showed a total increment by a factor of 1.4 from the largest to the smallest particles as compared to a factor of approx. 5.5 in DCB glucose release. If only the substrate surface area was affecting the extent of enzymatic hydrolysis, then increasing factors of comparable numerical sizes would be expected between particle sizes. Indeed, these results showed that yield differences between substrate particle sizes reduced when the material was pretreated; however, any real explanation to this phenomenon has not been proposed in the literature, but it seems likely that it could be related to biomass

compositional differences, exactly because these were also reduced when DCB was pretreated. The only exception from the overall trend was the differences in glucose yield obtained for particle size fractions [355;250] and [250;150] µm for pretreated DCB (Fig. 1). The discrepancy was caused by the relatively low glucose content in size fraction [355; 250] µm (Table 2), resulting in a false overestimation of the yield. The raw data did not show a higher degree of hydrolysis in this particular substrate fraction. As a result of these considerations, the obtained data seem to represent an effect of true differences in biomass composition in addition to increased surface area effects.

That the distribution of biomass into portions of more similarly composed materials will affect the enzymatic hydrolysis opens the possibility of targeting enzymatic hydrolysis even more. Still, the enzymatic treatments did not degrade the polysaccharide structure of corn bran significantly, even though all seemingly relevant enzyme activities were present. Obtaining more similarly composed fractions of substrate would simplify the picture and might thereby help to understand and break down the puzzle of the cell wall structure.

Theoretical Assessment of Successful Enzyme-Substrate Collisions

Available substrate surface and collision probability

Based on the results obtained, it was intriguing to attempt an assessment of the significance of surface area changes for the enzymatic hydrolysis. This was done by estimating the number of successful enzyme-substrate encounters, *i.e.* collisions between substrate particles and enzyme molecules resulting in reaction.

The main consequence of milling and sieving the substrate into different particle size fractions is that the total surface area and the number of particles increase with decreasing particle size. Table 6 contains an estimate of how the total substrate surface area changed with changing particle size, with all other things being equal, and an estimate of the maximum number of enzymes that might occupy the entire particle surface area. The calculations were done via a series of assumptions and equations (Table 5). One of the main assumptions behind the calculations was that the substrate particles could be represented by completely dense spherical-shaped structures that would pack as closely as possible in a cubic closest packed face centered cell (Zumdahl 1998) defining a unit cell of 4 particles (Eq. 1, Table 5). It was also assumed that the substrate density regardless of particle size was close to 1 g/mL when fully hydrated in the reaction.

The number of enzymes occupying the particle surface area was estimated by considering the maximum number of enzyme molecules that might be positioned next to each other without overlapping on the outer surface of the substrate particles. With respect to the enzymes, it was assumed that they had an average molecular weight of 80 kDa (Eq. 6, Table 5). Furthermore, it was assumed that each enzyme could be represented by cubic structures of 3x3x3 nm^3, where a surface area of 9 nm^2 could interact with the substrate.

The molecular size of glycosyl hydrolases varies greatly, but the molecular masses of the enzymes in the minimal enzyme mixture used in these hydrolysis experiments ranged from about 42 to 100 kDa (Agger *et al.* 2010; Sørensen *et al.* 2007). Generally, the molar mass of glycosyl hydrolases lay within the range of 25 to 125 kDa (Harris *et al.* 2010).

Table 5. Equations used to Estimate Successful Enzyme-Substrate Collisions (results in Table 6 and 7) *

Eq.#	
1	$V_{unit\,cell} = \left(l_{unit\,cell}\right)^3 \cdot 10^{-12} = \left(r \cdot \sqrt{8}\right)^3 \cdot 10^{-12}$
2	$A_{particle} = 4 \cdot \pi \cdot r^2 \cdot 10^{-12}$
3	$n_S = C_S \cdot \frac{4}{V_C} = C_S \cdot \frac{4}{\left(r\sqrt{8}\right)^3 \cdot 10^{-12}}$
4	$m_{particle} = \rho \cdot \frac{4}{3} \cdot \pi \cdot r^3 \cdot 10^{-18}$
5	$M_{sample} = X_k \cdot C_{DM}$
6	$n_e = \frac{m_e \cdot N_A}{MW_e}$
7	$N_S = \frac{m_{particle} \cdot n_S \cdot N_A}{MW_c} = \frac{\rho \cdot \frac{4}{3} \cdot \pi \cdot r^3 \cdot C_S \cdot 4 \cdot N_A}{MW_c \left(r\sqrt{8}\right)^3 \cdot 10^6} = \frac{\rho \cdot \frac{16}{3} \cdot \pi \cdot C_S \cdot N_A}{MW_C \left(\sqrt{8}\right)^3 \cdot 10^6}$
8	$N_{SA} = \frac{N_S}{A_{total}} = \frac{N_S}{n_S \cdot A_{particle}} = \frac{m_{particle} \cdot n_S \cdot N_A}{n_S \cdot A_{particle}} = \frac{\rho \cdot \frac{4}{3} \cdot \pi \cdot r^3 \cdot 10^{-18} \cdot N_A}{4 \cdot \pi \cdot r^2 \cdot 10^{-12} \cdot MW_C} = \frac{\rho \cdot r \cdot N_A}{3 \cdot MW_C \cdot 10^6}$
9	$X_i = X_k \cdot \frac{r_i^2}{r_k^2} \cdot \frac{n_{S,i}}{n_{S,k}}$

* Reactions are assumed to occur during 24 hours incubation (conditions as stated in the EXPERIMENTAL section). Substrate particles are assumed to be dense spheric structures that will pack as close as possible in a cubic closest packed face centered cell defining a unit cell of 4 particles (see text). It is also assumed that substrate particle density is $1 \cdot 10^6$ g/m^3 and that the substrate particle is completely made up of polysaccharides.

Table 5, continued. Equations in Table 5 above explained.

Eq.#	
1	$V_{unit\,cell}$ is volume of unit cell [mL]. $l_{unit\,cell}$ is length of one edge in the unit cell [µm] r is particle radius [µm]
2	$A_{particle}$ is surface area of spherical particle [m^2]. r is particle radius [µm]
3	n_S is number of substrate particles per sample [substrate particles/sample] C_S is substrate concentration [2% w/v DM]. r is particle radius [µm]
4	$m_{particle}$ is substrate particle mass [g]. r is particle radius [µm]
5	M_{sample} is total mass conversion during hydrolysis per sample [g] X_k is conversion in the k'th particle size [g/kg DM]. C_{DM} is total sample DM [$20 \cdot 10^{-6}$ kg]
6	n_e is number of enzyme molecules per sample [enzyme molecules/sample] m_e is enzyme mass loaded to each sample [12 mg]. N_A is Avogrado's number [$6.022 \cdot 10^{23}$ /mole]. MW_e is avr. molecular weight of an enzyme [80000 g/mol]
7	N_S is number of successful hits per substrate particle [hits/substrate particle] MW_c is the average molecular weight of a carbohydrate monomer
8	N_{SA} is total number of successful hits per particle surface area [hits/m^2]
9	Extrapolation of conversion from the conversion in the k'th particle (X_k) to the l'th particle (X_l) [g/kg DM]. r_l is radius of the l'th particle [µm]. r_k is radius of the k'th particle [µm] $n_{S,l}$ is the number of particles in the l'th size sample $n_{S.k}$ is the number of particles in the k'th size sample

In addition to assuming that the particles were exclusively made up of polysaccharides (hence, ignoring the lignin and protein contents), accordingly led to the assumption that the product of every successful enzymatic reaction is a monosaccharide with an equal chance of being a hexose or a pentose; in turn, an average molecular weight of 168 g/mol was used. Obviously, this particular heterogeneous substrate and the use of a multi-enzyme blend, including both processive and endo-acting enzyme activities, form a particular complex and unfortunate model case. Nevertheless, the principles of the calculations and the results may provide some important clues to the consequences of substrate particle size diminution on enzymatic deconstruction of insoluble biomass polysaccharides.

As expected, the number of enzymes that was calculated to interact with the substrate increased with increasing surface area (and diminishing particle size), in this case from 16 to 109 pmol (Table 6).

Table 6. Estimations of the Number of Substrate Particles, Substrate Surface Area, and Enzyme Loading Capacities of Different Corn Bran Substrate Particle Sizes. Estimations Calculated According to Equations Given in Table 5

Particle size (µm)	Radius (m)	Unit cell volume (mL)	Number of particles (#/sample)	Substrate surface area (m^2/sample)	Maximal number of enzymes (pmol/sample)
1000	$5.00 \cdot 10^{-4}$	$2.83 \cdot 10^{-3}$	28	$8.88 \cdot 10^{-5}$	16
710	$3.55 \cdot 10^{-4}$	$1.01 \cdot 10^{-3}$	79	$12.5 \cdot 10^{-5}$	23
355	$1.78 \cdot 10^{-4}$	$1.27 \cdot 10^{-4}$	632	$25.0 \cdot 10^{-5}$	46
250	$1.25 \cdot 10^{-4}$	$4.42 \cdot 10^{-5}$	1810	$35.6 \cdot 10^{-5}$	66
150	$0.75 \cdot 10^{-4}$	$9.55 \cdot 10^{-6}$	8380	$59.2 \cdot 10^{-5}$	109
Enzyme load*	0.12	mg/sample			
	$1.5 \cdot 10^{3}$	pmol/sample			

*Total enzyme loading as in enzymatic hydrolysis experiments: 6 mg enzyme protein/g DM.

When compared to the total enzyme load in each sample, however, it is evident that the number of free enzymes in solution was far greater than the available substrate surface (Table 6). Similar considerations have been described by Axelrod and Wang (1994). They introduced the "reaction-limited" receptor concept, leading to low reaction probability as the rate limiting process rather than limitations in reactant collisions. It can fairly easily be recognized that the number of reactant collisions are not limiting in this scenario either, since the number of individual substrate particles ranges from approximately 28 to 8400 and should be compared to an enzyme loading of $9 \cdot 10^{14}$ individual enzyme molecules, equivalent to $1.5 \cdot 10^{3}$ pmol (Table 6).

From the apparent difference in order of magnitude it seems obvious that the probability of substrate particles constantly colliding with an enzyme are very high; however, as described in a general receptor concept by Axelrod and Wang (1994), the

successful reactions are strongly dependent on thermodynamics on a micro-scale level possibly due to Brownian motions of the enzymes that may influence whether an effective binding occurs. Specifically for these kinds of enzymatically-catalyzed reactions, the kinetics and affinities for each enzyme are crucial, in particular in the case where a certain coordination between activities occur, *i.e.* in the case of endo-β-xylanase and β-xylosidase activities and endo-glucanase and β-glucosidase activities.

Successful enzyme hits; theoretical versus experimental conversion

Despite the many factors affecting enzymatic reactions and the complex kinetics of concerted enzymatic reactions, such as those involved in biomass polysaccharide deconstruction, it is intriguing to attempt to describe the events of successful reactions. If these enzymatic reactions are simplified to a system of single, independent reactions, and if it is assumed that every successful hit leads to the release of one monomeric component, *i.e.* in this case a monosaccharide, regardless of the actual mechanism (and disregarding that neither cellobiohydrolases, endo-glucanases, nor endo-xylanases catalyze the release of monomeric constituents), then certain estimations can be made that will provide insight into the enzymatic reaction dependency on particle size. It is therefore possible to estimate a total number of successful hits in every sample and a total number of successful hits per substrate surface area. The latter can be interpreted as a measure of the enzymatic hydrolysis efficiency. The concept of successful hits thus encompasses the collision between the enzyme and the substrate and accomplishment of the enzyme catalyzed reaction. When calculations are based on constant substrate dry matter (in each type of substrate particle size fraction), the number of successful hits must be constant between the different substrate samples, when the same mass of substrate dry matter is converted. When full conversion of all dry matter is assumed, then a correlation between total substrate surface area and the number of successful hits per substrate surface area can be deduced by the use of Eqs. 2, 3, 7, and 8 (Table 5, Table 7). This correlation is visualized in Fig. 2A. As explained from Eq. 7 (Table 5), the total number of successful hits is *independent* of particle size. In the example of total conversion the total number of successful hits is constant among different particle sizes. The plot (Fig. 2A) therefore shows that when the total substrate surface area decreases, the efficiency of the hydrolysis needs to increase significantly in order to still convert the same amount of substrate.

The full conversion example provides only limited insight into how hydrolysis depends on particle size, since the calculation assumes the same degree of hydrolysis for all particles. The quantitative assessment is valuable, however, for deriving an extrapolation expression for estimating the enzymatic conversion in an insoluble (biomass) substrate having different particle sizes – if, and only if – the differences in enzymatic hydrolysis resulting from differences in particle size are solely dependent on particle size. Based on such an extrapolation expression (Eq. 9 in Table 5), it is possible to estimate the conversion in all particle sizes if the conversion in one particle size is known. Table 7 shows such extrapolations together with the real observed conversion from experiments on DCB, Fig. 1A, and these are plotted in Fig. 2B as a function of particle size.

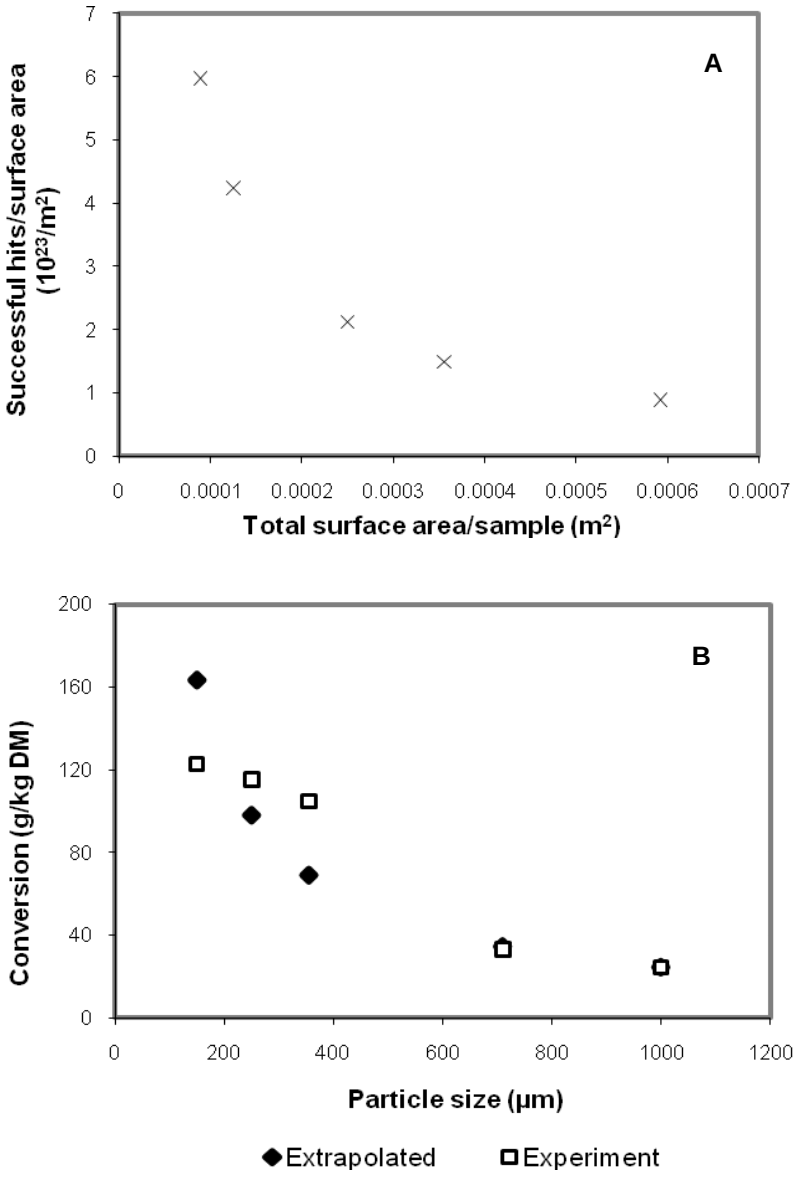

Fig. 2. A: Theoretically estimated number of successful hits per substrate surface area when assuming full conversion in all particle size fractions as a function of total substrate surface area. **B**: Biomass conversion as a function of particle size comparing extra-polated (♦) and experimental (□) data. Data calculated from equations in Table 5, and data in Figure 1A

It is apparent that the hydrolysis of the second largest particles (710 µm) followed the extrapolation based on particle size (the calculated and the experimentally obtained points even overlap), whereas particles of 355 and 250 µm in diameter were somewhat more efficient in the hydrolysis than expected from the calculations (Fig. 2B).

The conversions in these two particular particle sizes were thus higher than what could be expected if these were only dependent on the corresponding increase in surface area. Interestingly, the smallest particles of 150 µm seem to fall behind in the conversion

as compared to the extrapolated example (Fig. 2B, Table 7). This indicates that the enzymatic hydrolysis in these particles was hindered compared to what could be expected if (increased) surface area was the only parameter determining the degree of hydrolysis, despite the smallest particle sizes yielding the highest conversion (g/kg DM) (Table 7).

Table 7. Estimations of Number of Enzyme-Substrate Interactions Leading to a Successful Reaction, *i.e.* Number of Successful Hits (N_S) per Substrate Particle and Number of Successful Hits (N_{SA}) per Substrate Surface Area when Conversion was Extrapolated Based on Changes in Substrate Surface Area (upper part) and Experimentally Observed Conversion (Figure 1)*

Extrapolation of conversion			
Particle size (µm)	Conversion X (g/kg DM)	N_S	N_{SA}
1000	24.5	$1.75 \cdot 10^{18}$	$1.97 \cdot 10^{22}$
710	34.5	$2.47 \cdot 10^{18}$	$1.97 \cdot 10^{22}$
355	68.9	$4.94 \cdot 10^{18}$	$1.97 \cdot 10^{22}$
250	97.8	$7.02 \cdot 10^{18}$	$1.97 \cdot 10^{22}$
150	163.1	$11.7 \cdot 10^{18}$	$1.97 \cdot 10^{22}$
Experimentally observed conversion			
Particle size (µm)	Conversion X (g/kg DM)	N_S	N_{SA}
1000	24.5	$1.75 \cdot 10^{18}$	$1.97 \cdot 10^{22}$
710	33.1	$2.38 \cdot 10^{18}$	$1.90 \cdot 10^{22}$
355	104.6	$7.50 \cdot 10^{18}$	$3.00 \cdot 10^{22}$
250	115.2	$8.26 \cdot 10^{18}$	$2.32 \cdot 10^{22}$
150[a]	122.6	$8.79 \cdot 10^{18}$	$1.48 \cdot 10^{22}$

[a] Experimental conversion of substrate particle sizes <150 µm was not included.

* In the extrapolated example conversion on the largest particles (1000 µm) was set to be the same as the experimentally observed. All estimations were based on the reaction volume, substrate and enzyme concentrations as for Fig. 1. Estimates calculated from equations shown in Table 5.

DISCUSSION

The fact that some particles were hydrolyzed better under enzymatic hydrolysis than what could be expected based on the surface area might have several different causes. Firstly, the observed hydrolysis differences could be related to differences in biomass composition. For simplicity, the estimations were based on an assumption of uniform biomass composition between particle sizes; however, as already discussed, the biomass composition was not the same among the differently sized particles, and the differences in biomass composition could very well explain some of the observed differences in conversion between the particles. With the enzyme blend used in these experiments (Table 1), some biomass compositions were simply more easily hydrolyzed

than others. Another possible reason for accelerated hydrolysis in the mid-range particles might be that the surface area of the particles changed during hydrolysis with respect to physical appearance and crystallinity for cellulose in particular. This could promote binding of the enzymes to some parts of the substrate. Similar considerations were described in the erosion model suggested by Väljamäe *et al.* (1998).

The enzymes might be able to migrate from a non-productive binding site to a productive one without dissociating away from the substrate surface. This mechanism was proposed by Axelrod and Wang (1994) and would reduce the dimensionality of the reaction from 3D to 2D. In turn, this would greatly enhance the efficiency of the enzymatic reaction. The finding that the hydrolysis of the smallest particle size samples was less effective than estimated might be related to the biomass composition. As the particles became smaller, protein and possibly lignin would tend to accumulate, which could hinder the enzymatic hydrolysis. Previous results of milling and sieving wheat straw showed that the content of ashes and minerals tended to build up in the smallest particle size fraction, which might also be contributing to lower conversion (Pedersen and Meyer 2009).

The estimations employed were based on very simplified assumptions, but they turned out to match the experimentally obtained enzymatic hydrolysis results quite precisely. Unfortunately, the enzyme system in the present example does not obey the assumptions of being independent and releasing a monosaccharide from each successful reaction. Furthermore, several dynamics like molecular velocity and movement, changing reaction conditions as a result of hydrolysis, biomass heterogeneity, and particle surface structure and porosity were not accounted for in these estimations. Also, all enzymes were treated as if there were no differences in catalytic mode of action. Without a doubt, these factors do influence the enzymatic process. Despite the simplifications, the estimations nevertheless provide a new quantitative insight and represent a new methodology to understand the dependency of enzymatic reactions on a physical property such as changed substrate particle size and surface area. Such simplified illustrations might give clues to how enhanced yields of enzymatic reactions are achieved.

CONCLUSIONS

1. The data demonstrated that milling and sieving of corn bran created different particle size fractions that varied in monosaccharide composition and arabinoxylan substitution.
2. Enzymatic hydrolysis of different substrate particle sizes gave different yields.
3. The data suggested that the differences in yields after enzymatic hydrolysis treatment were influenced by differences in both substrate particle surface area and biomass composition.
4. Theoretical estimations of substrate particle-enzyme collisions supported that parameters other than surface area, *e.g.* biomass composition, affected the enzymatic hydrolysis, but also showed that the trends in enzymatic hydrolysis efficiency could be predicted by a collision-based model.

ACKNOWLEDGMENTS

Tomas Fernqvist, Risø DTU and Ove Skov Nielsen, Novozymes A/S are acknowledged for assisting in the pretreatment and destarching of the corn bran, respectively. The work was co-financed by Novozymes A/S and the Food Graduate School Denmark.

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Article submitted: April 6, 2012; Peer review completed: May 28, 2012; Revised version received: June 4, 2012; Accepted: June 9, 2012; Published: June 14, 2012.